Back to the past—forever young: cutting-edge biochemical and microbiological tools for cultural heritage conservation

Back to the past. Forever young: cutting-edge biochemical and microbiological tools for cultural heritage conservation

Roberto Mazzoli[1], Gabriella Giuffrida[2] and Enrica Pessione[1]

Affiliations

[1]Università di Torino - Dipartimento di Scienze della Vita e Biologia dei Sistemi, Via Accademia Albertina 13, 10123 Torino, Italy.

[2]CNR-ISPA, Largo P. Braccini 2, 10095 Grugliasco, Torino, Italy.

[1]To whom correspondence should be addressed: Enrica Pessione,
Department of Life Sciences and Systems Biology. Università of Turin. Via Accademia Albertina 13. 10123 Torino. Italy.

Tel. +39 011 6704644

Fax +39 011 6704508

E-mail: enrica.pessione@unito.it

ABSTRACT

Ancient documents and milestones of human history such as manuscripts and textiles are fragile and during aging undergo chemical, physical and biological deterioration. Among the different causes of damage, also human intervention plays a role since some restoration strategies proved to be transient and/or they generated further damage. Outdoor monuments undergo deterioration since they are exposed to pollution, weathering, microbial attack (giving rise to undesired pigmentation, discoloration or true dissolution, corrosion, and overall decay) as well as men-made damage (i.e. graffiti). This review article reports the best fitting strategies used to restore wall paintings, outdoor monuments, textiles and paper documents to their ancient beauty by employing "soft" bio-based approaches such as viable bacteria or suitable enzymes.

Key-words: immobilized enzymes, biocleaning, caseinase, collagenase, viable bacteria, graffiti, bioconsolidation

INTRODUCTION

Artworks may undergo a number of degradation and deterioration events, which widely vary depending on the specific artifact and the environment and conditions of conservation. These parameters may be extremely different if we consider, for instance, a book conserved at controlled temperature and humidity in a library or in a museum, or a stone statue or a cathedral, which are constantly exposed to weathering, pollution, microbial colonization (extensively reviewed in Mazzoli et al. 2018), vandalism acts, etc… It is worth reminding that damaging of artworks is sometimes the effect of previous restoration interventions which underwent deterioration themselves during time, as in the case of glues applied to consolidate wall paintings or ancient textiles (Beutel et al. 2002; Ferrari et al. 2017).

Cleaning and/or restoration of artworks by biotechnological approach has been performed by using enzymes or microorganisms or a combination of both strategies, depending on the specific artifact and issue (Figs. 1, 2; Table 1). Bio-based methods have a number of advantages over more consolidated techniques for artwork restoration (such as those using non-acqueous solvents, bleaching and mechanical treatments), because of their lower impact on the environment, reduced toxicity for operators and higher selectivity and safety for artworks themselves (Barbabietola et al. 2016). Enzymes are generally characterized by extremely high substrate specificity which allows high selective choice depending on the "damaging material" (e.g. proteins, polysaccharides, lipids) to treat/remove. Moreover, enzymes can be chosen whose catalytic activity is optimal at the most suitable pH/T ranges for treating a certain artwork thus reducing the application time (Germinario et al. 2017). Enzymes have been used in aqueous formulations, with or without a gel as sorbent, and in ionic liquids (Hrdlickova Kuckova et al. 2014). On the other hand, the use of enzymes may be limiting because of the relatively high amounts required, their relatively high cost, the need for controlled application conditions (e.g. pH and T) and for skilled operators (Barbabietola et al. 2016). Therefore, the use of microorganisms has sometimes been preferred, for instance when very resistant or complex deposit materials (i.e. mixture of heterogeneous substances) or very extended surfaces (e.g. the surface of a cathedral) needed to be removed/treated. In addition, the use of living microorganisms is necessary in the case of complex phenomena such as calcium carbonate deposition for the bioconsolidation of stone material (Dhami et al. 2014). As compared to enzymatic strategies, the use of living microorganisms for biocleaning of artworks is certainly less expensive and may require less controlled environmental conditions, although it is generally less selective (Webster and May 2006).

Biocleaning-biorestoration of paper documents and textiles

One of the most common causes of biodeterioration of ancient papers (e.g. books, documents) and textiles that are preserved in museums, libraries and archives are glues employed, with specific variations and modifications, to manufacturing or consolidating/restoring these artifacts (Barbabietola et al. 2016; Ferrari et al. 2017). In the case of paper artworks, glues have been used in manufacturing, such as for bonding and lining of prints, drawings, documents, which were mounted (partly or completely) on secondary support by means of glue spots, as well as for restoration (Barbabietola et al. 2016). As regards historical or ethnographic textiles, glues have mainly been used for restoration purposes, e.g. to fasten them to textiles or to solid (paper or wood) supports (Ahmed and Kolisis 2011). In the past, glues of either vegetal (i.e. starch) or animal (i.e. collagen and/or casein) origin have been used for these purposes (Barbabietola et al. 2016; Ferrari et al. 2017). Both animal and vegetal glues are made up of natural polymers, that is mainly proteins (i.e. collagen derived from the bones, skins, tendons and cartilage of mammalians or fish swimming bladder) or polysaccharides (i.e. amylose and amylopectin derived from different plants, such as potato, rice, corn or wheat), respectively (Barbabietola et al. 2016; Ferrari et al. 2017). Through aging, these glues undergo stiffening and thickening which may in turn generate distortions, tensions and discoloration, or form intricate layers that are very recalcitrant to being removed (Blüher et al. 1995; Gostling 1989). In the case of animal glues, humidity, temperature, UV radiation and pollutants can generate protein cross-linking and/or hydrolysis/oxidation of peptide bonds, while microbial metabolism produces acid molecules and pigmented spots (Barbabietola et al. 2016). Starch glue has been commonly used for ancient textile restoration (Ahmed and Kolisis 2011; Whaap 2007). After aging, starch paste is generally found in shrunk, cracked, rigid and brittle form, which cannot provide enough adhesion for effective support. In this form, it can cause heavy damage to ancient textiles because of concomitant embrittlement, hardening, yellowness and acidity of the latter. Furthermore, starch may be a source of contamination by amylolytic fungi and bacteria that contribute to textile decay over time, especially when a suitable degree of humidity supporting microbial growth is present.

Consequently, cleaning of glue residues is often a priority in the restoration of ancient paper or textile artworks. Current mechanical and chemical methods display serious drawbacks mainly related to aggressiveness towards material and/or toxicity for the restorers and/or the environment. Humidification (also called wet-cleaning) has been used to swell starch paste, however it generally needs long treatments which are unsuitable for paper or textile artifacts and is often insufficient for aged or hardened glues. Bio-based methods, i.e. the use of enzymes or microorganisms, have been shown to be a very efficient alternative in a number of cases (Ahmed and Kolisis 2011; Barbabietola et al. 2016; Ferrari et al. 2017).

Enzymes are certainly the most frequently used method for the treatment of glue-damaged paper (Banik et al. 2003; Corbi et al. 2005; DeSantis 1983; Sandrine 2002) or textiles (mainly linen, silk and cotton fabrics, so far) (Ahmed and Kolisis 2011; Ciatti et al. 2010) and several successful examples have been reported in the literature. For instance, trypsin has been used for detaching a compact block of leaves (Wendelbo 1976). Amylases and proteases have been employed for detaching graphics from their backings (De la Chapelle 2003; Segal and Cooper 1977). Very recently enzyme extracts with protease activity isolated from marine invertebrates have been used to remove aged/altered protein glue layers from the velinatura (Japanese paper bonded by animal glue) of ancient oil on canvas or from polychrome wood (Palla et al. 2016). This proved to be a cutting–edge strategy also useful to bio-clean fragile artworks such as wax sculptures. An additional advantage of these marine invertebrate-derived extracts is their antimicrobial activity useful to control bacteria/fungi growth (Palla and Barresi 2017). It is worth noting that application of enzymes in solution is not always suitable for paper or textile artworks, since it may involve artifact flooding with excess water which favors mold and fungi colonization and growth and thus causes further damage to the artifact (Ahmed and Kolisis 2011). Actually, water-dissolved α-amylase preparations have been applied locally either in solution (Ahmed and Kolisis 2011) (Fig. 1a-c; Table 1) or as poultice (Bott 1990; Chapman 1986; Shibayama and Eastop 1996) for the bio-restoration of starch-glue treated textiles. In general, the use of immobilized enzymes is preferable for these applications. Theoretically, all enzymes can be immobilized. However, it is worth reminding that the immobilization yield and the enzyme efficiency should be determined for each specific enzyme and immobilization strategy, to limit the loss of enzyme and catalytic activity. A ready-to-use poultice of amylolytic enzymes, called Albertina Kompresse, was developed by an Austrian group for removing non-swellable starch-based glue from graphic artworks of albums of the "Albertina" graphic collection in Vienna (Schwarz et al. 1999). Phytagel™ was used for lowering and controlling water content in enzyme solutions (Iannucelli and Sotgiu 2009) used for cleaning -etchings depicting the Chinea of Clemente VIII, dating 1598. Gellan hydrogel-immobilized α-amylases have been developed for removing starch paste from ancient paper documents (Mazzuca et al. 2014). A gellan-immobilized bacterial α-amylase has been recently used to clean a wool shroud dating back to the Coptic period from a starch glue that had been used in the 1950s to temporary consolidate the textile (Ferrari et al. 2017) (Fig. 1d-h; Table 1). After selection of the suitable enzyme (among those commercially available) and optimization of the conditions for enzyme immobilization, the cleaning of the back of the two fragments (about 4 m^2 of textile) composing the tunic was completed in 160 h of work (Ferrari et al. 2017). A recent study (Barbabietola et al. 2016) has described the first attempt to bio-cleaning ancient paper from animal glue by using living bacteria (Table 1). To this aim, non-pathogenic, non-spore-forming and non-

cellulolytic *Ochrobactrum sp.* TNS15E was used after immobilization on agar gel. This bacterial pack was used to remove glue layers from paper documents dating back to the 17-18th century. Four-hour treatment was sufficient to clean the cellulose fibers from glue, as confirmed by both colorimetric and scanning electron microscopy (SEM) analyses.

Apart from paper/textile biocleaning from glue, it is worth reminding the case of aged drying-oil stains on both ancient paper and textiles. During drying and aging, double bonds of unsaturated fatty acids are oxidized by oxygen in the air, giving rise to multiple products which include nonhomogeneous polymeric network of triacylglycerides which may be hard to being removed (Ahmed et al. 2010; Blüher et al. 1997). Lipases such as that of *Candida cylindracea* have been used to clean such aged drying-oily stains from paper documents (Blüher et al. 1997) or textiles such in the case of a coptic tunic (Ahmed et al. 2010) (Table 1).

Biorestoration/biocleaning of stone artworks

Durable stone (e.g. marble and/or limestone) has been used for the construction of a multitude of artworks and monuments all along the human history and all over the world including the Egyptian Pyramids, the Greek and Roman temples and theaters, the European Cathedrals and the Taj Mahal in India. Unfortunately, all of them have been suffering from progressive deterioration caused by both biotic and abiotic agents (Dhami et al. 2014). These numerous factors have led to stone dissolution, staining or color alteration, surface alteration, bio-corrosion and transformations into smaller sized crystals, etc… (Chand and Cameotra 2011). In the recent decades, microbial biofilm production, deposition of organic (such as residual hydrocarbons and other organic pollutants in dust) and inorganic compounds (formation of nitrate and sulfate alterations such as the black crusts) have been among the main deterioration events (Antonioli et al. 2005; Di Pippo et al. 2009; Fernandes 2006; Warscheid and Braams 2000). Actually, limestone mainly consists of the most stable polymorph of calcium carbonate, i.e. calcite, (with only a small content of aragonite) but is very porous and hydrophilic. This makes limestone very susceptible to water flush (especially acid rain), environmental pollutants and physical, chemical and biological (e.g. microorganisms) weathering (Dhami et al. 2014). Therefore, survival of many cultural and historical assets is in threat. One of such examples is the cave of Lascaux in southwest France which is considered the best conserved prehistorical example of human wall painting art (they are also named the paleolytic Cappella Sistina). In this site, infection of *Fusarium sp.* and other molds have deteriorated the floor and banks of the main chamber (Rosenbaum 2006), but also autotrophic organisms such as green algae have produced green pigments because of the intense illumination and improved CO_2 availability related

to visitors (Bastian et al. 2010). Martin-Sanchez and co-workers (2012) have extensively studied the effectiveness of biocides in the cave biocleaning. Fungicides had been intensively applied to treating the cave since 2001 that were essentially targeted to remove *Fusarium sp* but obtained little success. In 2008, a new biocide treatment was planned due to black stains that appeared on the cave surfaces. DGGE analysis on these stains showed the presence of *Ochroconis lascauxensis*. This result demonstrated the ineffectiveness of the previous biocide treatments on the long time which appeared to favor colonization by other fungal strains and therefore increase fungal diversity. Later, i.e. in 2010, fungal communities were quite different from those detected in 2008, since the main identified strain was a yeast belonging to the *Herpotrichiellaceae* family. It is clear that careful preliminary study on the possible advantages and disadvantages of applying biocides in subterranean environments is required (Martin-Sanchez et al. 2012).

Many attempts have been made to fix such structural damages by application of traditional conservative treatments such as organic and inorganic chemicals (Lazzarini and Laurenzi Tabasso 1986). However, these agents have been most often low effective, in spite of their aggressiveness (which, on the other side, has led the concomitant risk of further damaging the artwork). Moreover, these strategies involve the use of high amounts of solvents, which are finally discarded in the environment creating problems of sustainability (Dhami et al. 2014). Alternatively, physical treatments such as laser cleaning have been used, but at significantly higher costs (Germinario et al. 2017). Furthermore, all these treatments have short duration effects thus requiring repeated interventions with relevant economic issues for public and private conservation agencies. Overall, conventional treatment methods have therefore proved to be unsatisfactory.

The shortcomings of conventional strategies have encouraged research in new conservation and remediation strategies based on biological methods (Fernandes 2006). As for the treatment of other type of artifacts, bio-based restoration approaches for stone materials are characterized by lower cost, toxicity and aggressiveness towards the artworks (Germinario et al. 2017). As described in the following sections, bio-based methods have been used to remove different degradation products from stone monuments, wall paintings, and marble statues (Germinario et al. 2017), including deposits of environmental pollutants (Margesin et al. 2011) and synthetic polymers present in adhesives (Giordano et al. 2018) as well as in paints used by graffiti writers (Sanmartin et al. 2014) (Fig. 2; Table 1). In addition, biocleaning has been performed on stone artworks suffering from inaccurate or aged restoration intervention (Beutel et al. 2002; Antonioli et al. 2005) (Fig. 2; Table 1).

Removal of sulfate and nitrate alterations

One of the most important causes of decay of calcareous stones is the conversion of calcium carbonate into calcium sulfate (gypsum) mainly caused by acid rains (i.e. containing significant amounts of sulfuric and nitric acid) (Ranalli et al. 1997). For instance, the genesis of "gypsum crusts" on the surface of such porous material can engender following fractures of the underlying stone. When calcium sulfate salts are accumulated together with atmospheric particles (pollen, dust, spores, small particles of smog) the so called "black crusts" are formed (Fig. 2a). For the removal of sulfates from artistic stoneworks, procedures based on the use of sulfate-reducing bacteria have been reported. Different bacterial strains of the genus *Desulfovibrio* (e.g. *D. desulfuricans* and *D. vulgaris*) (either pure or in mixed cultures) have been applied under anaerobic conditions to marble samples directly or after adhesion to a sepiolite matrix (Ranalli et al. 1997). The use of sepiolite promoted sulfate removal on both simulated samples and real marble statue artifacts. On the latter, 81 % sulfate removal was obtained after 36 h treatment (Ranalli et al. 1997). Actually, *D. desulfuricans* (Ranalli et al. 1997) and *D. vulgaris* (Ranalli et al. 1997; Cappitelli et al. 2007a; Alfano et al. 2011) have been widely employed in restoration/removal of sulfate crusts from other artifacts (Table 1). Use of biotechnological cleaning on durable stone monuments can sometimes comply with multiple types of deterioration such as described by Cappitelli et al. (2007b). In this study, the sulfate-reducing bacterium *D. vulgaris subsp. vulgaris* ATCC 29579 was employed to remove the black crust found on marble of the Milan Cathedral (Italy). Compared to chemical cleaning (i.e. ammonium carbonate-EDTA) strategy, the microbial-catalyzed approach resulted in more homogeneous removal of the deposits and higher preservation of the original surface (Cappitelli et al. 2007b) (Table 1). Both chemical and biological treatments converted gypsum (i.e. calcium sulfate) to calcite (i.e. calcium carbonate), allowing consolidation. However, the chemical strategy also formed undesirable sodium sulfate while the use of *D. vulgaris* did not (Cappitelli et al. 2007b). Nonetheless, biological removal of sulfates may require quite long application periods, depending on the thickness of the crust. A recent study has demonstrated that this period can be greatly shortened and general efficiency of biocleaning can be significantly improved by combining the use of sulfate-reducing bacteria with a non-biological strategy, e.g. the use of a non-ionic detergent (Troiano et al. 2013) (Fig. 2a, b; Table 1). This combined strategy shortened application times of about 38-70 % depending on the specific artifact to be cleaned (Troiano et al. 2013).

Another consequence of acid rains (and of the action of living microorganisms) is the deposit of calcium nitrate salts on stone buildings and wall paintings (Dhami et al. 2014). Here, again, pollution increases the presence of various nitrogen oxides in the atmosphere that in turn may react with rain water and form nitrous and, more abundantly, nitric acid which then reacts with stone and replaces calcium carbonate with calcium nitrate (Dhami et al. 2014). Different strains of

Pseudomonas spp. have been recently applied for removing calcium nitrate salts from two stone monuments. Agar-entrapped *Pseudomonas stutzeri* DSMZ 5190 has been used for the biocleaning of nitrate efflorescence from wall paintings located in the lunettes of the central vault of the Santos Juanes church in Valencia, Spain (Bosch-Roig et al. 2013) (Fig. 2c-e; Table 1). The chosen strategy proved to be extremely efficient allowing to remove 92 % of the precipitates in 90 minutes. *Pseudomonas pseudocaligenes* KF707 has been used to remove nitrate salts from the tuff stone surfaces of the 12th century Matera Cathedral, Italy (Alfano et al. 2011) (Table 1). Here, carbogel-entrapped bacteria were applied to the Cathedral walls and allowed quick removal of the surface nitrate deposits, since 55 % of the nitrate salts were "cleaned" after 24 h.

Bioconsolidation

Apart from interventions aimed at removing superficial deposits and/or crust from stone monuments, the use of calcifying bacteria offers a chance to consolidate decayed building structures and materials. This application, sometimes also called microbial geotechnology (intending microbial-based technology for civil structures) actually mimics nature since many carbonate rocks have been cemented by carbonate precipitation induced by microorganisms during Earth geological cycles. This relatively novel and environmental-friendly technology has been studied for at least 20 years and has already been used for protecting and/or restoring different decayed construction materials/artifacts (Dhami et al. 2012; 2013).

Calcium carbonate precipitation is a chemical process (described by equation 1) which is influenced by four main factors, i.e. calcium concentration, amount of dissolved inorganic carbon (DIC), availability of nucleation sites and pH (Hammes and Verstraete 2002).

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3 \qquad Eq.1$$

$$K_{SP,Calcite,25°C} = [Ca^{2+}]\,[CO_3^{2-}] = 4.8 \times 10^{-9} \qquad Eq.2$$

Calcium carbonate precipitation occurs when the product of concentrations of Ca^{2+} and CO_3^{2-} is higher than the solubility product (K_{SP}) of calcium carbonate (Eq. 2). However, the amounts of CO_3^{2-} in a given system depends on both the amount of DIC (which in turn depends on several parameters such as temperature and partial pressure of carbon dioxide) and pH. Because of the high number of parameters that may contribute to control calcium carbonate precipitation, different bacteria, isolated from different habitats, are able to create local micro-environments that induce such phenomenon (Hamilton 2003) (Fig. 3). The four main groups of microorganisms that may influence calcification are: (i) photosynthetic organisms such as cyanobacteria and algae, (ii) sulfate reducing bacteria

responsible for dissimilatory reduction of sulfates, (iii) organisms utilizing organic acids, and (iv) organisms that are involved in nitrogen cycle either by ammonification of amino acids/nitrate reduction or hydrolysis of urea (Stocks-Fischer et al. 1999; Hammes and Verstraete 2002; Jargeat et al. 2003) (For an exhaustive review please refer to the study of Dhami et al. 2014). The precipitation of carbonates by bacteria through urea hydrolysis is the most straightforward and easily controlled mechanism of microbial induced calcium carbonate precipitation since it produces high amounts of carbonates and an alkaline environment (Dhami et al. 2014). Boquet et al. (1973) firstly demonstrated the precipitation of calcium carbonate by soil bacteria under laboratory conditions (Fig. 3). At that time, several *Bacillus spp.* and *Pseudomonas aeruginosa* were shown to form calcite crystals. In 1990, Adolphe et al. patented the concept of using calcifying microorganisms to treat artificial surfaces and founded the "Calcite Bioconcept" company. However, the first *in situ* application of bioconsolidation was carried out in Thouars (France) on the tower of the Saint Médard Church by using *Bacillus cereus* only in 1993 (Le Metayer-Levrel et al. 1999) (Table 1). Although this application was judged as successful, some drawbacks were: the need to regularly repeat the treatment (for instance, each 10 years); the presence of natural pigments in the nutritional medium of *B. cereus* which co-precipitated with calcium carbonate thus giving the new stone layer a light persistent coloring; the formation of endospores and a thin biofilm of *Bacillus sp.* For these reasons, Rodriguez-Navarro et al. (2003) proposed to replace *Bacillus sp.* with a Gram-negative, non-pathogenic soil bacterium, i.e. *Myxococcus xanthus*. Tiano et al. (1999) studied the effect of *Micrococcus spp.* and *Bacillus subtilis* on Pietra di Lecce bioclastic limestone. Variations in the kind of bacteria used and the methods for bacterial cell delivery to the stone surface have been tested by different Authors with variable success (Daskalakis et al. 2014; Dhami et al. 2014; Helmi et al. 2016; Micallef et al. 2016). Recently, the use of indigenous calcifying bacteria for re-inoculation of stone monuments has been proposed as an alternative strategy for bioconsolidation (Jroundi et al. 2017). However, microbiologically driven calcification remains more complex than chemical methods, since microbial activity depends on many factors such as temperature, pH, concentrations of donors and acceptors of electrons and concentration and diffusion rates of nutrients and catabolites. Hence, the use of microbial calcification at large scales has not been always encouraged since it may be hard to manage (Dhami et at. 2014). Also the cost of media required for bacterial growth may be a significant economic limit of this approach (Achal et al. 2009; 2010).

Biorestoration of wall paintings

As previously mentioned, also stone artworks may suffer from inaccurate or aged restoration strategies. This was the case of two wall paintings covered by animal glue layers during restoration interventions which have been restored using different bio-based approaches as described by Beutel et al. (2002) and Antonioli et al. (2005), respectively (Table 1; Fig. 2). The first study concerns medieval wall paintings called "Falcon hunt-meeting of the living and the dead" located in St. Alexander church in Wildeshausen, Germany. These paintings were suffering from severe peeling off from the roughcast surface (Beutel et al. 2002) (Table 1). Actually, this is a common problem of wall paintings in medieval churches of the Northern Europe since they have long been treated by application of casein layers to stabilize them (Beutel et al. 2002). As for other glue-like matrices, aging in addition to climate effects causes progressive hardening and stiffening of casein layers thus causing an even more drastic peeling off of the painted parts from the surface (Beutel et al. 2002). This problem was fixed by removing aged casein layers through application of a selected microbial serine-protease (Alcalase 2.5 DX-L). The enzyme was covalently immobilized onto an epoxide-functionalized cellulose acetate membrane (Beutel et al. 2002). By 2D fluorescence monitoring of the tryptophan exposed by casein hydrolysis, it could be estimated that 30-minute treatment was sufficient for substantial removal of the casein layers from the mural painting.

The case of "Conversion of S. Efisio and battle" fresco by Spinello Aretino at the monumental Cemetery of Pisa (Italy) was even more complicated (Fig. 2f, g; Table 1). Because of weathering and other environmental aging, this fresco needed to be restored and for this purpose it was removed from the wall surface by using the tear-off technique in the 1980s. Firstly, the fresco was covered with a gauze that was sticked by applying an animal glue (mixed with high concentrations of formaldehyde, as antimicrobial agent). Once the glue had been hardened, the fresco was detached from the wall. Unfortunately, the fresco was then forgotten until 2000s in a storeroom, so that, traditional application of protease mixtures were unable to remove the gauze (Antonioli et al. 2005). Because of the long time of storage, it is likely that the presence of formaldehyde had promoted the formation of a resistant net of cross-linked proteinaceous material, which was very recalcitrant to protease catalysis. However, the selection of a bacterial strain (i.e. *Pseudomonas stutzeri*) able to grow on chips of insoluble glue harvested from the "clothed" fresco (and hence possessing the right collagenase enzymes), finally helped to solve the problem. Actually, it was then possible to use this bacterial strain (i.e. cotton strips impregnated with live *Pseudomonas stutzeri* were applied) directly on the fresco and completely degrade the glue layer and remove the cloth from the fresco after only 12 hour treatment (Fig. 2f, g).

Biocleaning of graffiti and synthetic adhesives

Stone monuments are not only aggressed by atmospheric events or microorganisms but, unfortunately, also by vandalism acts, including painting by unauthorized graffiti. Graffiti materials have a complex chemical composition that comprises synthetic polymers such as acrylics, alkyds and nitrocellulose, and several additives (Germinario et al. 2017). Quick removal of graffiti is an important issue, since the fresher the graffiti, the easier is their removal. Here again, bio-based (through enzymes or microorganisms) removal of synthetic materials is an emerging strategy which has already shown good results in a number of cases.

One of the first examples of acrylic material removal by means of bio-based approach has been described by Bellucci et al. (1999). Here, a lipase has been used to eliminate aged acrylic ParaloidB72 resin from a 15th century tempera painting on panel and a 19th century oil painting on canvas. In both artworks, the presence of surface layers containing ParaloidB72 was the result of previous restoration interventions occurred in the early 1970s and in the 1980s, respectively (Bellucci et al. 1999). Cleaning was likely achieved via hydrolysis of the ester groups of the acrylate and methacrylate units contained in the synthetic resin leading to free carboxylic acid groups. This reaction therefore generated more hydrophilic products which facilitated acrylic resin removal by aqueous cleaning systems (Bellucci et al. 1999). Bio-based removal of acrylic materials is particularly advantageous for treating painting where the use of traditional methods, e.g. organic solvents, would likely remove also original paint layers. However, the same approach can be employed also to treat stone materials. For instance, Germinario et al. (2017) tested different lipases in oil-in-water micro-emulsions for the removal of acrylic marker pen inks from unglazed ceramic surfaces. Very recently, Palla and co-workers (2017) successfully detached a canvas layer glued by the same acrylic ParaloidB72 resin to a mosaic sample by applying a gelled (3% Klucel G) enzymatic solution with esterase activity. The bio-removal was very fast, only 40 minutes at room temperature (Palla et al. 2017). The same research group also reported the removal of adhesive-tape glue residues present on specific areas of an acrylic paint on canvas. They used a microemulsion of Velvesil Plus® (a surfactant used in the cosmetic field) containing an esterase derived by a marine organism that proved to be active even at a temperature lower than 30°C (whereas most commercially available enzymes have an optimum temperature of 37°C) (Palla et al. 2016). These authors demonstrated that the enzymatic solution can be merged into the Velvesil Plus® gel (without any negative effect on enzyme activity) and easily applied to remove the undesired layers. The contact between enzyme and the layer to be removed was obtained by gently moving the microemulsion, by a soft-brush for 5 minutes (Giordano et al. 2018). As regards the use of living microorganisms, *D. desulfuricans* has proved able

to degrade nitrocellulose-based paints (Giacomucci et al. 2012), while the use of different bacterial strains has been tested for the bio-cleaning of acrylic polymers used in the restoration field (Troiano et al. 2014).

CONCLUSION

This report demonstrates that it is possible to face cultural heritage damage due to aging, weathering, pollution or wrong restoration interventions by using bacteria or purified enzymes suitably immobilized to contain the risk of employing aqueous solutions. Among the wide range of enzymes commercially available those displaying a good catalytic activity at low temperatures (lower than 30°C) are very promising since they can be applied also to fragile items. The microbial world as well as the marine environment seem to be good candidates to be explored for finding such enzymes. This field is promising also to find, in the future, a solution to contain microbial deterioration (Mazzoli et al. 2018) thus avoiding the use of acids, solvents and surfactants (dangerous for the artworks, the art restorers and the environment) for instance by using enzyme- or bacteriocin-mediated bacterial competition. In this case, the safety and effectiveness of the microorganisms employed is mandatory and the need for control and analysis before and after treatments strongly recommended. Interdisciplinary approaches and collaborations between art conservators and biotechnologists, biochemists and microbiologists is the essential requisite to preserve objects that state the immense creativity of artists and the high value of human history.

COMPLIANCE WITH ETHICAL STANDARDS

Funding: this work was financially supported by “Ricerca Locale-ex 60%” of the Turin University.

Conflict of interest: all the Authors declare that they do not have any conflict of interest.

Ethical approval: this article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

Achal V, Mukherjee A, Basu PC, Reddy MS (2009) Lactose mother liquor as an alternative nutrient source for microbial concrete production by *Sporosarcina pasteurii.* J Ind Microbiol Biotechnol 36:433-438.

Achal V, Mukherjee A, Reddy MS (2010). Biocalcification by *Sporosarcina pasteurii* using Corn steep liquor as nutrient source. J Ind Biotechnol 6:170-174.

Adolphe JP, Loubière JF, Paradas J, Soleilhavoup F (1990) Procédé de traitement biologique d'une surface artificielle. European Patent 90400G97.0. (after French patent 8903517, 1989), (France)

Ahmed HE, Gremos SS, Kolisis FN (2010) Enzymatic Removal of the Oily Dirt from a Coptic Tunic using the Enzyme Lipase. Journal of Textile and Apparel, Technology and Management 6 (3)

Ahmed HE, Kolisis FN (2011) An investigation into the removal of starch paste adhesive from historical textiles by using the enzyme of alpha-amylase. J Cult Herit 12:169-179.

Alfano G, Lustrato G, Belli C, Zanardini E, Cappitelli F, Mello E., Sorlini C, Ranalli G (2011) The bioremoval of nitrate and sulfate alterations on artistic stone work: the case-study of Matera Cathedral after six years from the treatment. Int Biodeterior Biodegrad 65:1004-1011.

Antonioli P, Zapparoli G, Abbruscato P, Sorlini C, Ranalli G, Righetti PG (2005) Art-loving bugs: the resurrection of Spinello Aretino from Pisa's cemetery. Proteomics 5:2453-2459.

Banik G, Cremonesi P, De La Chapelle A, Montalbano L (2003) Nuove Metodologie nel Restauro del Materiale Cartaceo. Il Prato, Padova, Italy.

Barbabietola N, Tasso F, Alisi C, Marconi P, Perito B, Pasquariello G, Sprocati AR (2016) A safe microbe-based procedure for a gentle removal of aged animal glues from ancient paper. Int Biodeterior Biodegrad 109:53-60.

Bastian F, Jurado V, Nováková A, Alabouvette C, Saiz-Jimenez C (2010) The microbiology of Lascaux cave. Microbiology 156:644-652.

Bellucci R, Cremonesi P, Pignagnoli G (1999) A preliminary note on the use of enzymes in conservation: the removal of aged acrylic resin coatings with lipase. Stud Conserv 44:278-281.

Beutel S, Klein K, Knobbe G, Königfeld P, Petersen K, Ulber R, Scheper T (2002) Controlled enzymatic removal of damaging casein layers on medieval wall paintings. Biotechnol Bioeng 80:13-21.

Blüher A, Grube A, Bornscheuer U, Banik G (1997) A reappraisal of the enzyme lipase for removing drying-oil stains on paper. The Paper Conservator 21: 37-47.

Blüher A, Haller U, Banik G, Thobois E (1995) The application of carbopol poultices on paper objects. Restaur Int J Preserv Libr Arch Mater 16:234-247.

Boquet E, Boronat A, Ramos-Cormenzana A (1973). Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. Nature 246:527–529.

Bosch-Roig P, Regidor Ros JL, Estellés RM (2013) Biocleaning of nitrate alterations on wall paintings by *Pseudomonas stutzeri*. Int Biodeterior Biodegrad 84:266-274.

Bott G (1990) Amylase for starch removal from a set of 17th century embroidered panels. Conservator 14: 23-29.

Cappitelli F, Principi P, Pedrazzani R, Toniolo L, Sorlini C (2007a) Bacterial and fungal deterioration of the Milan Cathedral marble treated with protective synthetic resins. Sci Total Environ 385:172-181.

Cappitelli F, Toniolo L, Sansonetti A, Gulotta D, Ranalli G, Zanardini E, Sorlini C (2007b) Advantages of using microbial technology over traditional chemical technology in removal of black crusts from stone surfaces of historical monuments. Appl Environ Microbiol 73:5671-5675.

Chand T, Cameotra SS (2011) Geomicrobiology of heritage monuments and artworks: mechanisms of biodeterioration, bioconservation strategies and applied molecular approaches In: Bioremediation: Biotechnology, Engineering and Environmental Management, Nova Science Publishers, New York, NY, pp. 233-266.

Chapman V (1986) Amylase in a viscous medium–textile applications. Conservator 10:7-11.

Ciatti M, Conti S, Fineschi C, Nelson J, K, Pini S (2010) Il ricamo in or nué su disegno di Raffaellino del Garbo. Aspetti storico-stilistici, tecnici, minimo intervento e conservazione preventiva. OPD Restauro 22:81-116.

Corbi M, Di Franco ML, Severi M, Santarelli ML, Filetici P (2005) Biotecnologie applicate alla rimozione di colle d'amido nel restauro della carta e del libro. Proceedings of the 3rd IGIIC National Congress -Lo Stato dell'Arte, 98-101.

Daskalakis MI, Magoulas A, Kotoulas G, Katsikis I, Bakolas A, Karageorgis AP, Mavridou A, Doulia D, Rigas F (2014) *Cupriavidus metallidurans* biomineralization ability and its application as a bioconsolidation enhancer for ornamental marble stone. Appl Microbiol Biotechnol 98:6871-6883.

De La Chapelle A (2003) Utilizzo degli enzimi nel restauro delle opere grafiche policrome. In: Cremonesi P (ed) Materiali tradizionali ed innovativi nella pulitura dei dipinti e delle opere policrome mobili. Atti del Primo Congresso Internazionale Colore e Conservazione - materiali e metodi nel restauro delle opere policrome mobili, Piazzola sul Brenta (PD), Italy.

De Santis P (1983) Some observation on the use of enzymes in paper conservation. J Am Inst Conserv 1:7-27.

454 Dhami NK, Mukherjee A, Reddy MS (2012) Biofilm and microbial applications in biomineralized
455 concrete. In: Jong Seto (ed) Advanced Topics in Biomineralization, InTech, New York, NY, pp. 137-
456 164.

457 Dhami NK, Mukherjee A, Reddy MS (2013) Biomineralization of calcium carbonates and their
458 engineered applications: a review. Front Microbiol 4:314.

459 Dhami NK, Reddy MS, Mukherjee A (2014) Application of calcifying bacteria for remediation of
460 stones and cultural heritages. Front Microbiol 5:304.

461 Dick J, De Windt W, De Graef B, Saveyn H, Van der Meeren P, De Belie N, Verstraete W (2006)
462 Bio-deposition of a calcium carbonate layer on degraded limestone by *Bacillus species*.
463 Biodegradation 17:357-367.

464 Di Pippo F, Bohn A, Congestri R, De Philippis R, Albertano P (2009) Capsular polysaccharides of
465 cultured phototrophic biofilms. Biofouling 25:495-504.

466 Fernandes P (2006) Applied microbiology and biotechnology in the conservation of stone cultural
467 heritage materials. Appl Microbiol Biotechnol 73:291-296.

468 Ferrari M, Mazzoli R, Morales S, Fedi M, Liccioli L, Piccirillo A, Cavaleri T, Oliva C, Gallo P, Borla
469 M, Cardinali M, Pessione E (2017) Enzymatic laundry for old clothes: immobilized alpha-amylase
470 from *Bacillus sp.* for the biocleaning of an ancient Coptic tunic. Appl Microbiol Biotechnol 101:7041-
471 7052.

472 Germinario G, van der Werf ID, Palazzo G, Ros JLR, Montes-Estelles RM, Sabbatini L (2017)
473 Bioremoval of marker pen inks by exploiting lipase hydrolysis. Prog Org Coat 110:162-171.

474 Giacomucci L, Toja F, Sanmartín P, Toniolo L, Prieto B, Villa F, Cappitelli F (2012) Degradation of
475 nitrocellulose-based paint by *Desulfovibrio desulfuricans* ATCC13541, Biodegradation 23:705-716.

476 Giordano A, Rotolo V, Di Carlo E, Palla F (2018) Novel esterases for cultural heritage. X AIAr
477 National Congress, Torino, Italy, February 14-17, 2018.

478 Gioventù E, Lorenzi PF, Villa F, Sorlini C, Rizzi M, Cagnini A, Griffo A, Cappitelli, F. (2011)
479 Comparing the bioremoval of black crusts on colored artistic lithotypes of the Cathedral of Florence
480 with chemical and laser treatment. Int Biodeterior Biodegrad 65:832-839.

481 Gostling K (1989) Bookbinders and adhesives: part 2. New Bookbind 9:30-39.

482 Hamilton WA (2003). Microbially influenced corrosion as a model system for the study of metal
483 microbe interactions: a unifying electron transfer hypothesis. Biofouling 19:65–76

484 Hammes F, Verstraete W (2002). Key roles of pH and calcium metabolism in microbial carbonate
485 precipitation. Rev Environ Sci Biotechnol 1:3–7

486 Helmi FM, Elmitwalli HR, Elnagdy SM, El-hagrassy AF (2016). Biomineralization Consolidation of
487 Fresco Wall Paintings Samples by *Bacillus sphaericus*. Geomicrobiol J 33:625-629.

488 Hrdlickova Kuckova S, Crhova Krizkova M, Pereira CL, Hynek R, Lavrova O, Busani T, Branco LC,
489 Sandu IC (2014) Assessment of green cleaning effectiveness on polychrome surfaces by MALDI-
490 TOF mass spectrometry and microscopic imaging. Microsc Res Tech 77:574-585.

491 Iannucelli, S., Sotgiu, S., 2009. La pulitura superficiale di opera grafiche a stampa con gel rigidi. In:
492 Progetto Restauro, 49. Il Prato, Padova, pp. 15-24.

493 Jargeat P, Rekangalt D, Verner MC, Gay G, Debaud JC, Marmeisse R, Fraissinet-Tachet L. (2003)
494 Characterisation and expression analysis of a nitrate transporter and nitrite reductase genes, two
495 members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma*
496 *cylindrosporum*. Curr Genet 43:199-205.

497 Jroundi F, Schiro M, Ruiz-Agudo E, Elert K, Martín-Sánchez I, González-Muñoz MT, Rodriguez-
498 Navarro C (2017) Protection and consolidation of stone heritage by self-inoculation with indigenous
499 carbonatogenic bacterial communities. Nat Commun 8:279.

500 Lazzarini L, Laurenzi Tabasso M (1986) Il Restauro della Pietra. CEDAM, Padua, Italy.

501 Le Metayer-Levrel G, Castanier S, Orial G, Loubiere JF, Perthuisot JP (1999) Applications of
502 bacterial carbonatogenesis to the protection and regeneration of limestones in buildings and historic
503 patrimony. Sediment geol 126:25-34.

504 Margesin R, Płaza GA, Kasenbacher S (2011) Characterization of bacterial communities at heavy-
505 metal-contaminated sites. Chemosphere 82:1583-1588.

506 Martin-Sanchez PM, Nováková A, Bastian F, Alabouvette C, Saiz-Jimenez C (2012). Use of biocides
507 for the control of fungal outbreaks in subterranean environments: the case of the Lascaux Cave in
508 France. Environ Sci Technol 46:3762-3770.

509 Mazzoli R, Giuffrida MG, Pessione E (2018) Back to the past. Find the guilty: microorganisms
510 involved in the biodeterioration of archeological and historical items. Appl Microbiol Biotechnol (In
511 press).

512 Mazzuca C, Micheli L Cervelli E, Basoli F, Cencetti C, Coviello T, Iannuccelli S, Sotgiu S, Palleschi
513 A (2014) Cleaning of paper artworks: development of an efficient gel-based material able to remove
514 starch paste. ACS Appl Mater Inter 6: 16519-16528.

515 Micallef R, Vella D, Sinagra E, Zammit G (2016) Biocalcifying *Bacillus subtilis* cells effectively
516 consolidate deteriorated Globigerina limestone. J Ind Microbiol Biotechnol 43:941-952.

517 Palla F, Barresi G (2017) Biotechnology and Conservation of Cultural Heritage, Springer
518 International Publishing, Switzerland.

519 Palla F, Barresi G, Chisesi RM, Cammarata M, Di Carlo E, Drago S, Giordano A, Lombardo G,
520 Rotolo V, Schiavone S, Stampone G, Trapani MR (2017) Innovative and Integrated Strategies: Case
521 Studies. In: Palla F, Barresi G (eds) Biotechnology and Conservation of Cultural Heritage, Springer
522 International Publishing, Switzerland, pp 85-100.

523 Palla F, Barresi G, Giordano A, Schiavone S, Trapani MR, Rotolo V, Parisi MG, Cammarata M
524 (2016) Cold-active molecules for a sustainable preservation and restoration of historic-artistic
525 manufacts. Int J Conserv Sci 7: 239-246

526 Ranalli G, Chiavarini M, Guidetti V, Marsala F, Matteini M, Zanardini E, Sorlini C (1997) The use
527 of microorganisms for the removal of sulphates on artistic stone works. Int Biodeterior Biodegrad
528 40:255-261.

529 Rodriguez-Navarro C, Rodriguez-Gallego M, Ben Chekroun K, Gonzalez-Munoz MT (2003)
530 Conservation of ornamental stone by *Myxococcus xanthus* induced carbonate biomineralization. Appl
531 Env Microbiol 69:2182-2193.

532 Rosenbaum MS (2006). Field Meeting Report: Bromfield Sand and Gravel Pit, nr Ludlow,
533 Shropshire, led by Ed Webb, 22nd April 2005. Proceedings of the Shropshire Geological Society
534 11:12-17.

535 Sandrine D (2002) Enzyme used for adhesive removal in paper conservation: a literature review. J
536 Soc Arch 2:187-195.

537 Sanmartìn P, Cappitelli F, Mitchell R (2014) Current methods of graffiti removal: a review, Constr
538 Build Mater 71:363-374.

539 Schwarz I, Bluher A, Banik G, Thobois E, Maurer KH (1999) The development of a ready-for-use
540 poultice for local removal of starch paste by enzymatic action. Restaurator 20:225-244.

541 Segal J, Cooper D (1977). The use of enzymes to release adhesives. Pap Conservator 2:47-50.

542 Shibayama N, Eastop D (1996) Removal of flour paste residues form a painted banner with alpha
543 amylase. Conservator 20:53-64.

544 Stocks-Fischer S, Galinat JK, Bang SS (1999) Microbiological precipitation of $CaCO_3$. Soil Biol
545 Biochem 31:1563-1571.

546 Tiano P, Biagiotti L, Mastromei G (1999) Bacterial bio-mediated calcite precipitation for
547 monumental stones conservation: methods of evaluation. J Microbiol Methods 36:139-145.

548 Troiano F, Gulotta D, Balloi A, Polo A, Toniolo L, Lombardi E, Daffonchio D, Sorlini C, Cappitelli
549 F (2013) Successful combination of chemical and biological treatments for the cleaning of stone
550 artworks. Int Biodeterior Biodegrad 85:294-304.

551 Troiano F, Vicini S, Gioventù E, Lorenzi PF, Improta CM, Cappitelli F (2014) A methodology to
552 select bacteria able to remove synthetic polymers. Polym Degrad Stab 107:321-327.

553 Warscheid T, Braams J (2000) Biodeterioration of stone: a review. Int. Biod. Biodegr. 46:343-368.

554 Webster A, May E (2006) Bioremediation of weathered-building stone surfaces. Trends Biotechnol
555 24:255-260.

556 Wendelbo O (1976) The Use of Proteolytic Enzymes in the Restoration of Paper and Papyrus.
557 University Library of Bergen.

558 Whaap F (2007) The treatment of two Coptic tapestry fragments. V&A Conservation Journal 55:11-
559 13.

560

Figure Legends

Fig. 1. Biocleaning of ancient textiles from starch glue. Pictures refer to biocleaning of a historical carpet dating back to the Ottoman period and exhibited in the museum of the Faculty of Applied Arts, Helwan University, Egypt (a, b, c) (modified from Ahmed and Kolisis 2011) and a coptic tunic dating back to the 5-6th century A.D. and exposed at the Egyptian Museum, Turin, Italy (d, e, f, g, h) (modified from Ferrari et al. 2017). b, c Detail of the carpet before (b) and after (c) the α-amylase treatment. e-h, Details of the Coptic tunic before (e, g) and after (f, h) the α-amylase treatment.

Fig. 2. Biorestoration of stone monuments. a, b, Detail of a marble statue dedicate in 1921 by Lina Arpesani to the poetess Anna Zuccari and located in the Monumental Cemetery of Milan (Italy). The black crusts (a) affecting the statue were cleaned (b) by using sulfate-reducing *Desulfovibrio vulgaris* (modified from Troiano et al. 2013). c, d, e Cleaning of the wall painting in the lunette of the Santos Juanes church, Valencia, Spain from nitrate salt efflorescence by means of agar gel-entrapped *Pseudomonas stutzeri.* Pictures represent the fresco area before (c), during (d) and after biocleaning (modified from Bosch-Roig et al. 2013). f, e biorestoration of the Spinello Aretino fresco "Conversion of S. Efisio and battle" in the Monumental Cementery of Pisa (Italy). f For animal glue removal. Cotton strips impregnated with live *Pseudomonas stutzeri* were applied leading to fresco biocleaning (g) (modified from Antonioli et al. 2005)

Fig. 3. Calcifying bacteria. Colonies of 6 different strains of *Bacillus sphaericus* and *Bacillus lentus* on agar plates during calcium carbonate deposition are shown (Dick et al. 2006).

Table 1. Some of the most significant examples of biorestoration/biocleaning of artworks described in the present study.

Type of artwork	Specific artwork (specimen)	Historical period of the specimen	Issue	Biorestoration/biocleaning strategy	Reference
Paper	Graphic artworks from albums (Graphic Collection Albertina, Vienna, Austria)	XIX century A.D.	Removal of aged starch glue	Gel-entrapped α-amylase	Schwarz et al. 1999
Paper	Paper documents of the Genoese Republic (Central Institute for Graphic Arts, Rome, Italy)	XVII-XVIII century A.D.	Removal of aged animal glue	Agar-immobilized *Ochrobactrum sp.* TNS15E	Barbabietola et al. 2016
Textile	Coptic tunic (Greek-Roman Museum, Alexandria, Egypt)		Removal of aged oily stains	Lipase from *Candida cylindracea*	Ahmed et al. 2010
Textile	Carpet (Museum of the Faculty of Applied Arts, Helwan University, Egypt)	Ottoman period	Removal of aged starch glue	α-amylase from *Aspergillus oryzae*	Ahmed and Kolisis 2011
Textile	Coptic tunic (Egyptian Museum, Turin, Italy)	V-VI century A.D.	Removal of aged starch glue	Gellan immobilized α-amylase from *Bacillus sp.*	Ferrari et al. 2017
Stone monument	Milan Cathedral (Italy)	XV century A.D.	Removal of black crust	Sulfate-reducing *Desulfovibrio vulgaris* ATCC 29579	Cappitelli et al. 2007b
Stone monument	Florence Cathedral (Italy)	XV century A.D.	Removal of black crust	Carbogel-entrapped sulfate-reducing *Desulfovibrio vulgaris* ATCC 29579	Gioventù et al. 2011
Stone monument	Matera Cathedral (Italy)	XII century A.D.	Removal of nitrate and sulphate crusts	Carbogel entrapped nitrate-reducing *Pseudomonas pseudoalcaligenes* KF707 and sulfate-reducing *Desulfovibrio vulgaris* ATCC 29579	Alfano et al. 2011
Stone monument	Stone column and marble statue, Monumental Cemetery (Milan, Italy)	XX century A.D.	Removal of black crust	*Desulfovibrio vulgaris* ATCC 29579 plus non-ionic detergent	Troiano et al. 2013

Stone monument	Saint Médard Church, (Thouard, France)	XII century A.D.	Limestone bioconsolidation	*Bacillus cereus*	Le Metayer et al. 1999
Wall paintings	Wall paintings of the lunettes of the central vault, Santos Juanes church (Valencia, Spain)	XVII-XVIII century A.D.	Removal of calcium nitrate salt efflorescence	Agar-entrapped *Pseudomonas stutzeri* DSMZ 5190	Bosch-Roig et al. 2013
Wall paintings	Falcon hunt-Meeting of the living and the dead, St. Alexander church (Wildeshauen, Germany)	XIV century A.D.	Removal of aged casein layers	Covalently immobilized protease (Alcalase 2.5 DX-L)	Beutel et al. 2002
Wall paintings	Conversion of S. Efisio and battle by Spinello Aretino, Monumental Cemetery (Pisa, Italy)	XIV century A.D.	Removal of aged formaldehyde-reated animal glue	Cotton strips impregnated with *Pseudomonas stutzeri* A29	Antonioli et al. 2005
Painting on panel	The Visitation with St. Joseph, St. Zacharias and Four Angels	XV century A.D.	Removal of acrylic resin	Lipase from *Candida cylindracea*	Bellucci et al. 1999
Painting on canvas	Portrait of a man	XIX century A.D.	Removal of acrylic resin	Lipase from *Candida cylindracea*	Bellucci et al. 1999

Fig. 1.

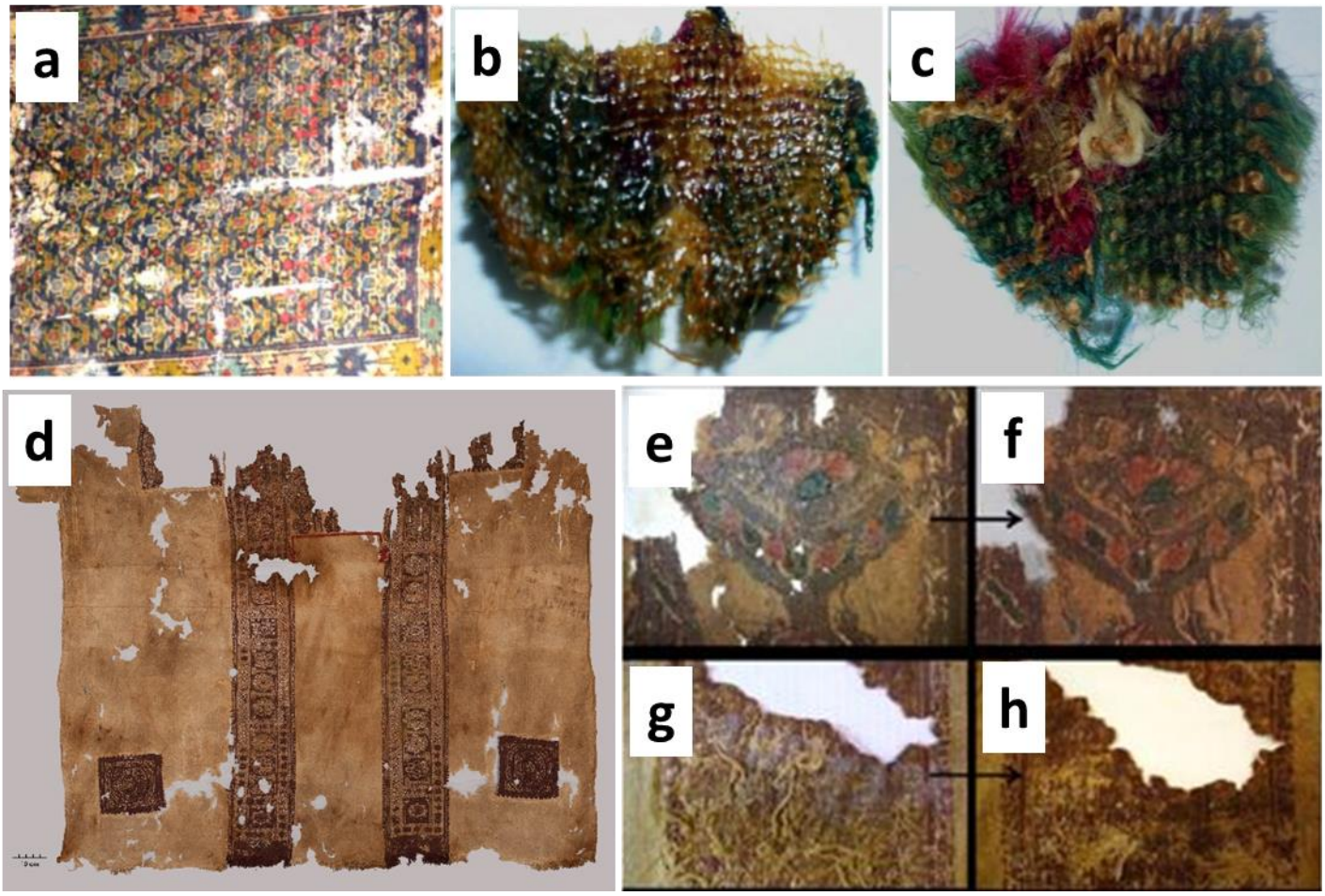

Fig. 2.

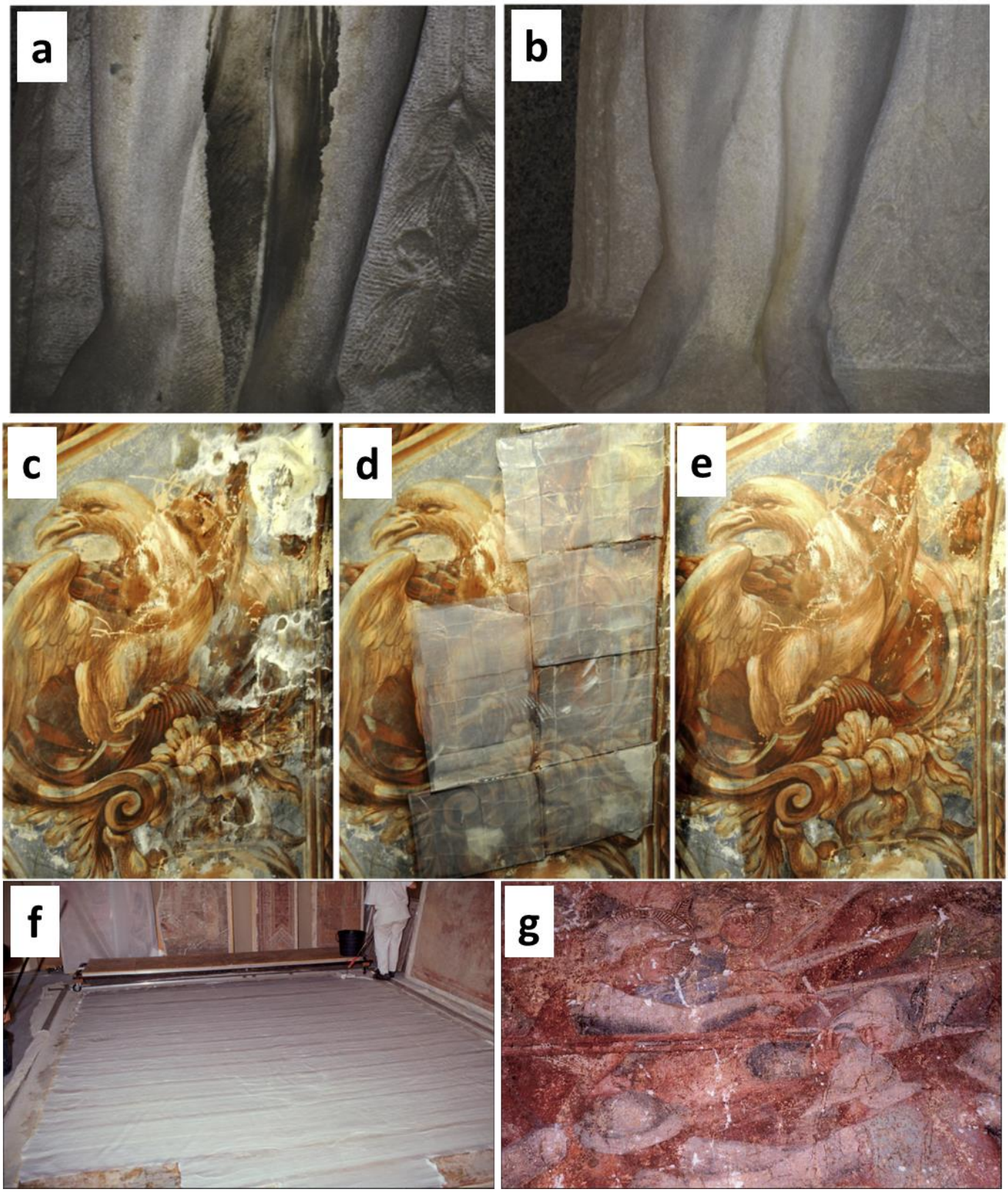

Fig. 3.